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1) Mol Microbiol 1998 Apr;28(1):131-41
Aromatic ligand binding and intramolecular signalling of the phenol-responsive sigma54-dependent regulator DmpR.
O'Neill E, Ng LC, Sze CC, Shingler V.

2) J Bacteriol 1994 Dec;176(24):7550-7
An aromatic effector specificity mutant of the transcriptional regulator DmpR overcomes the growth constraints of Pseudomonas sp. strain CF600 on para-substituted methylphenols.
Pavel H, Forsman M, Shingler V.

3) Biodegradation 1994 Dec;5(3-4):219-36
Genetics and biochemistry of phenol degradation by Pseudomonas sp. CF600.
Powlowski J, Shingler V.

4) J Bacteriol 1994 Aug;176(16):5052-8
Cross-regulation by XylR and DmpR activators of Pseudomonas putida suggests that transcriptional control of biodegradative operons evolves independently of catabolic genes.
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Thank you,
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Cross-Regulation by XylR and DmpR Activators of *Pseudomonas putida* Suggests that Transcriptional Control of Biodegradative Operons Evolves Independently of Catabolic Genes

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The *Pu* promoter of the toluene degradation plasmid pWW0 of *Pseudomonas putida* drives expression of an operon involved in the sequential oxidation of toluene and *m*- and *p*-xylenes to benzoate and toluates, respectively. Similarly, the *Po* promoter of plasmid pVI150 controls expression of an operon of *Pseudomonas* sp. strain CF600 which is required for the complete catabolism of phenol and cresols. These promoters, which both belong to the σ^{54}-dependent class, are regulated by their cognate activators, XylR and DmpR, respectively. XylR and DmpR are homologous proteins, and both require aromatic compounds as effector molecules for activity. However, these two proteins respond to different profiles of aromatic compounds. The activity of each promoter in the presence of the heterologous regulator was monitored using *lacZ* and *luxAB* reporter systems. Genetic evidence is presented that the two activators can functionally substitute each other in the regulation of their corresponding promoters by binding the same upstream DNA segment. Furthermore, when coexpressed, the two proteins appear to act simultaneously on each of the promoters, expanding the responsiveness of these systems to the presence of effectors of both proteins. Potential mechanisms for the occurrence of evolutionary divergence between XylR and DmpR are discussed in view of the DNA sequence similarities among *Pu*, *Po*, and a third XylR-responsive promoter, *Ps*.

Many strains of *Pseudomonas* species and related gram-negative bacteria are able to use a variety of unusual aromatic chemicals, including many xenobiotic compounds, as carbon sources (15). This ability is frequently determined by large, low-copy-number plasmids that express one or more catabolic operons. The toluene degradation (TOL) plasmid, pWW0, of *Pseudomonas putida* mt-2 (3) and the pVI150 plasmid of *Pseudomonas* sp. strain CF600 (36, 40) are two prototypes. Plasmid pWW0 encodes two operons (Fig. 1) as follows: the first codes for the oxidative transformation of toluene and *m*- and *p*-xylenes to the corresponding benzoate and toluates, respectively (*upper* pathway operon), and the second encodes the subsequent metabolism of the carboxylic acids via a *meta* ring cleavage pathway to tricarboxylic acid cycle intermediates (*lower* pathway operon [15, 37]). The catabolic plasmid pVI150 encodes a single gene cluster, the *dmp* operon, for the catabolism of phenol and cresols via hydroxylation and a subsequent *meta* cleavage pathway (Fig. 1). The *Pu* promoter of the *upper* TOL operon (7, 21, 25, 28) and the *Po* promoter of the *dmp* operon (39) are two examples of σ^{54}-dependent promoters. Their respective regulators, XylR and DmpR, activate transcription from their cognate promoters upon exposure of the cells to distinct pathway substrates (2, 41), such as *m*-xylene in the case of XylR and phenol in the case of DmpR. These two proteins belong to the NtrC family of transcriptional activators, members of which are composed of distinct functional domains in a fashion reminiscent of that found in eukaryotic enhancer-binding proteins (32). Despite regulating expression of functionally different operons and being responsive to distinct effector profiles, XylR and DmpR are remarkably similar throughout their entire sequences (39), suggesting a common evolutionary origin. This homology has permitted us to experimentally address one question concerning the acquisition of a transcriptional control by degradative pathways, namely, whether regulators and the cognate DNA sequences to which they bind are recruited by catabolic systems together or independently. Our results clearly show that XylR and DmpR can efficiently cross-activate each other's promoter in spite of the different organizations of the *Pu* and *Po* regions. These data support the idea that genes for σ^{54}-dependent activators may have evolved along with discrete binding sequences as regulatory modules that end up controlling transcription of unrelated pathways.

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MATERIALS AND METHODS

Strains, plasmids, and general procedures. The relevant strains and constructions used in this work are listed in Table 1. Recombinant DNA methods were carried out according to published protocols (27). Predetermined deletions at the *Pu* promoter region were introduced through site-directed mutagenesis (26) and were confirmed by DNA sequencing. Transposon vectors carrying different insertions were integrated into the chromosomes of target bacteria, as described elsewhere (8). Broad-host-range plasmids were introduced into *Pseudomonas* strains by electroporation (41) or by mobilization from *Escherichia coli* by triparental matings (8) with the helper strain *E. coli* HB101 (RK600).

Activity assays. β-Galactosidase (β-Gal) levels in cells permeabilized with chloroform and sodium dodecyl sulfate were determined (29). The linearity of the β-Gal assays within the range of cell densities and the times of reaction with *o*-nitro-

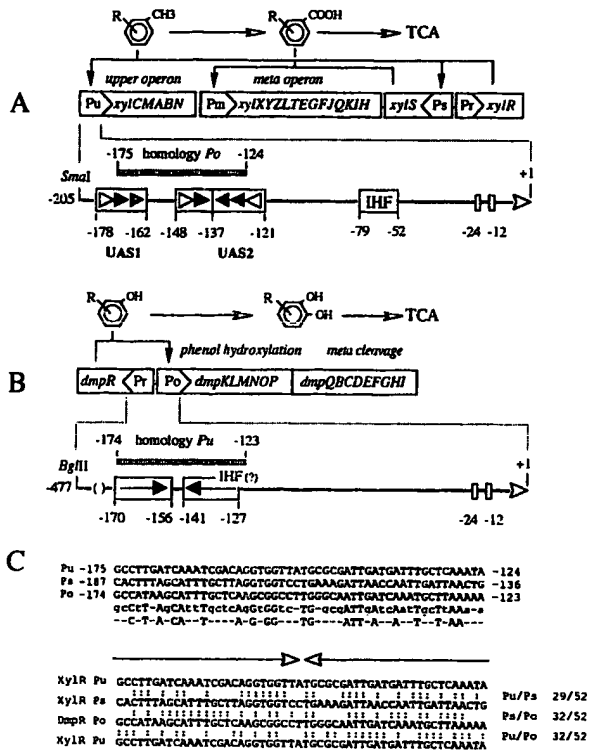

FIG. 1. (A) The regulatory cascade of the *xyl* genes in the TOL plasmid pWW0. In the presence of *upper* pathway substrates like *m*-xylene, the *upper* operon promoter *Pu* and the *xylS* promoter *Ps* are activated by XylR (22) in combination with σ^{54}-RNAP. Subsequently, an excess of XylS product or XylS bound to its effectors (i.e., substrates of the *meta* pathway) activates *Pm*. Note that there is no physical continuity between the *upper* and the *meta* operons (3, 15, 28). Below the scheme of the pathway, the *Pu* promoter region is expanded, showing the relevant flanking DNA. This includes UASs for XylR, the sequences between −12 and −24 recognized by σ^{54}-RNAP, and an IHF-binding site located within the intervening region. Upon binding this target DNA sequence in *Pu*, IHF induces a sharp DNA bend that brings into close contact the prebound σ^{54}-RNAP and the activator protein attached to the distant UAS (7). Repeated DNA sequences within the UAS are indicated by arrows (7). The portion of the *Pu* sequence which displays maximum nucleotide sequence similarity to *Po* is shown as a horizontal hatched bar. (B) Regulation of the pVI150-encoded *dmp* operon. The phenol- and cresol-responsive *dmpR* gene product activates transcription of the divergently transcribed *dmp* operon from the *Po* promoter. A subset of the *dmp* genes is involved in phenol hydroxylation, while the rest encode enzymatic activities of the *meta* cleavage pathway for dissimilation of the catechol intermediate. The C2,3O encoded by *dmpB* is homologous to other *meta* ring cleavage enzymes (12, 18). The *Po* promoter region is expanded below the scheme of the *dmp* pathway. Relevant portions of the sequence are pinpointed, including a long imperfect inverted repeat, the region of homology to *Pu*, and a potential IHF-binding site (39). (C) DNA sequence homology among the upstream regions of *Pu*, *Po*, and *Ps*. The sequences on the top align the regions of maximum nucleotide sequence similarity found in *Pu*, *Po*, and *Ps*, the last being the other XylR-dependent TOL promoter (see above). The same sequences are shown below as imperfect inverted repeats, and the numbers of matching bases between the different promoter regions are indicated.

phenyl-β-D-galactopyranoside (ONPG) were verified in all cases. Data for β-Gal activity are the averages of duplicate samples from a minimum of three independent experiments, values for which were within a variation of 15%. Light emission resulting from expression of the *luxAB* genes was measured using an LKB Luminometer 1250 set at 1 V as described elsewhere (34). Data for light emission are the averages of triplicate determinations from two independent experiments that gave values that differed by less than 20%. The specific activities (in units per milligram of protein) of the *dmpB*-encoded catechol-2, 3-dioxygenase (C2,3O) of crude extracts were determined as previously described (33). Cells from 2 to 40 ml of culture, depending on the optical density at 600 nm (OD_{600}), were harvested, washed in ice-cold 0.1 M phosphate buffer (pH 7.4), resuspended in 250 μl of the same buffer, and then disrupted by sonication. Cell debris was removed by centrifugation, and the supernatants were used as crude extracts. The variability between duplicate determinations did not exceed 10%.

Construction and chromosomal integration of *Pu-lacZ*. To faithfully monitor *Pu* activity, a monocopy gene dosage transcriptional *lacZ* gene fusion was constructed by first cloning a 312-bp *Eco*RI-*Bam*HI *Pu*-containing fragment from pEZ9 (7) in pBK16 (24). The resulting *Pu-lacZ* fusion plasmid, pBK16*Pu*, was introduced by conjugation in a *P. putida* strain harboring a chromosomally located minitransposon that provides homology to the sequences flanking the insertion in pBK16*Pu*. Double recombination between the plasmid and the minitransposon sequences results in the generation of a chromosomal *Pu-lacZ* fusion, which was readily identified by using vector selection markers (24). An equivalent insertion of a *lacZ* transcriptional fusion to *PuΔUAS* (a *Pu* derivative from which the upstream activating sequences which span the binding site for XylR had been deleted) was made by cloning the *Eco*RI-*Bam*HI restriction fragment of pFH15 (Table 1) into the *lacZ* vector pUJ8 (6). The resulting *PuΔUAS-lacZ* fusion was excised as a 4-kb *Not*I fragment and cloned in the transposon delivery plasmid pUT/mini-Tn*5* Sm (6) for further insertion into the chromosome of *P. putida* KT2442 as previously described (8). Irrespective of the method used, the chromosomal insertions with the *lacZ* constructs are organized such that the fusions are transcriptionally shielded upstream by the Ω element and downstream by a strong T7 terminator. For XylR-DmpR competition assays, a transposon, mini-Tn*5* XylR/*Pu-lacZ* (5), was used to generate a monocopy *Pu-lacZ* fusion identical to that described above but accompanied by a 2.4-kb DNA segment of the TOL plasmid spanning the *xylR* gene expressed under the control of its native constitutive promoter (5).

Construction and integration of the *Po-luxAB* fusion. Activity of the *Po* promoter in monocopy gene dosage was monitored through light emission resulting from expression of the *luxAB* genes of *Vibrio harveyi*. For this, we constructed a specialized *Pseudomonas* reporter strain in which a *Po-luxAB* reporter cassette was engineered as follows. A 2.8-kb *Sal*I-to-*Sma*I fragment from pVI360 (41), which carries a 478-bp *Po* promoter region (positions −477 to +2 with respect to the transcription initiation site) controlling expression of the promoterless *luxAB* genes, was cloned between the *Sal*I and *Eco*RV sites of pBluescript SK(+), in which an additional *Not*I site had been introduced in place of the *Xho*I site. The resulting construct allowed excision of the fusion as a 2.8-kb *Not*I fragment that was subsequently cloned into pUT/mini-Tn*5* Sm (6) and inserted into the chromosome of *P. putida* KT2440 as described elsewhere (8).

Induction conditions. The different *P. putida* strains were grown overnight at 30°C in Luria-Bertani (LB) medium supplemented, when required, with adequate antibiotics to retain a plasmid of interest. The cultures were diluted 1:200 in the same prewarmed medium and were grown in duplicate with good aeration to an OD_{600} of 0.1 to 0.5, as indicated for each

TABLE 1. Bacteria and plasmids used in this study

Strain or plasmid	Relevant genotype and characteristic(s)	Reference or origin
E. coli K-12		
CC118	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA thi-1 rpsE rpoB argE*(Am) *recA1*	17
CC118*supF*	CC118 with a chromosomal insertion; mini-Tn*5* Cm *supF*	24
CC118λ*pir*	CC118 lysogenized with λ*pir* phage	17
S17-1λ*pir*	Tp^r Sm^r *recA thi hsdRM*$^+$ RP4::2-Tc::Mu::Km Tn*7* λ*pir* phage lysogen	8
HB101	Sm^r *recA thi pro leu hsdRM*$^+$ (*E. coli* K-12/*E. coli* B hybrid)	27
P. putida		
KT2440	Prototrophic; reference strain	K. N. Timmis
KT2442	Prototrophic; Rif^r derivative of KT2440	17
KT2442*hom. fg.*	Km^r; KT2442 inserted with a DNA segment homologous to the flanking sequences of pBK16	24
SF05	KT2442 with chromosomal insertion; ΩSm/Sp *Pu-lacZ*	This work
SF05Δ	KT2442 with chromosomal insertion; mini-Tn*5* Sm/Sp *PuΔUAS-lacZ*	This work
207	KT2440 with chromosomal insertion; mini-Tn*5* Sm/Sp *Po-luxAB*	41
CNB3	KT2442 with chromosomal insertion mini-Tn*5* XylR/*Pu-lacZ*	This work
CF600	Wild-type strain; contains *dmp*$^+$ plasmid pVI150; Hg^r; growth on phenols and cresols as the sole carbon source	36
Plasmids		
pUT/mini-Tn*5* Sm	Ap^r Sm^r/Sp^r; R6K*oriV* RP4*oriT*, mini-Tn*5* Sm transposon vector delivery plasmid	6
pCNB3-*lacZ*	Ap^r Sm^r; R6K*oriV* RP4*oriT*; pUT/mini-Tn*5* XylR/*Pu-lacZ*	5
pUT/*PuΔUAS*	Sm^r/Sp^r; R6K*oriV*; RP4*oriT*; pUT/mini-Tn*5* Sm inserted with the 4-kb *Not*I fragment of pUJ*PuΔUAS*; *PuΔUAS-lacZ* transcriptional fusion	This work
pUT/*Po-lux*	Sm^r/Sp^r; R6K*oriV*; RP4*oriT*; pUT/mini-Tn*5* Sm inserted with a 2.8-kb *Not*I fragment containing *Po-luxAB* fusion	41
pVI360	Ap^r; *Po-luxAB* reporter plasmid	41
RK600	Cm^r; ColE1*oriV*; RK2*mob*$^+$ *tra*$^+$	24
pUJ8	Ap^r; *trp*::*lacZ* promoter probe vector, *lacZ* fusion sequence flanked by *Not*I sites	6
pUC18 Not	Ap^r; multiple cloning site vector; polylinker flanked by *Not*I sites	17
pVI361	Ap^r; DmpR gene cloned in the *Eco*RV site of pBS(+) as a 4.3-kb *Sma*I fragment from pVI150	This work
pUC-DmpR	Ap^r; DmpR gene cloned as an *Eco*RI-*Kpn*I 4.4-kb fragment of pVI361 in pUC18Not	This work
pKT231	Km^r Sm^r; broad-host-range plasmid (RSF1010 derivative)	10
pFH51	Km^r; pKT231 inserted with *dmpR*$^+$ as a 4.4-kb *Eco*RI-*Bam*HI fragment from pUC-DmpR	This work
pTK19	Km^r; pKT231 inserted with *xylR*$^+$ as a 2.4-kb *Hpa*I fragment of TOL plasmid pWW0	5
pEZ9	Ap^r; pUC18 inserted with a 312-bp *Eco*RI-*Bam*HI fragment spanning the entire *Pu* promoter	7
pCG2	Ap^r; ColE1*ori*; M13*ori*; vector for site-directed mutagenesis	31
pGC2*Pu*	Ap^r; pCG2 inserted with the 312-bp *Eco*RI-*Bam*HI fragment of pEZ9 including the *Pu* promoter sequence	This work
pFH15	Ap^r; same as pCG2*Pu* but with an *Eco*RI site created by site-directed mutagenesis at position −106 of the *Pu* sequence	This work
pBK16	Sm^r/Sp^r, *trp*::*lacZ* promoter probe vector, *supF*-suppressible amber codons in *aadA* and *lacZ* sequences	24
pBK16*Pu*	Sm^r/Sp^r; pBK16 inserted with the *Pu*-containing 312-bp *Eco*RI-*Bam*HI fragment of pEZ9	This work
pUJ*PuΔUAS*	Ap^r; pUJ8 inserted with the 208-bp *Eco*RI-*Bam*HI fragment of pFH15 containing *PuΔUAS*	This work

experiment. Subsequently, cultures were exposed to saturating vapors of the XylR effector (toluene or xylenes) and/or to a final concentration of 2.5 mM DmpR effectors (phenol or cresols). Under these conditions, the concentration of effector molecules was found to result in maximum activation of each of the reporter systems used. The cultures were then further grown as specified in each case, and aliquots were sampled at the indicated time points.

RESULTS

XylR and DmpR can replace each other for the activation of their cognate promoters. To determine whether the similarity between the XylR and DmpR regulators was sufficient to detect some level of cross-regulation between their respective promoters, we used the reporter strain *P. putida* 270 (*P. putida* KT2440::mini-Tn*5* Sm *Po-luxAB*), which contains a chromosomal *Po-luxAB* fusion. This strain harboring either pTK19, an RSF1010 derivative which determines constitutive expression of the *xylR* gene from its own promoter, or pFH51, an equivalent construct expressing *dmpR* from its native promoter, was subjected to induction assays with a range of substrates of the TOL *upper* pathway and the *dmp* pathway. The data shown in Fig. 2 clearly demonstrate that the response of the *Po* promoter was totally shifted from TOL substrates to *dmp* substrates and vice versa by changing the regulator encoded by the resident plasmid. This result is somewhat surprising because, in spite of the homology between the two proteins, the organization of the functional DNA elements within the *Po* promoter is different from that of the *Pu* promoter (Fig. 1). To examine whether such responsiveness to the two regulators (and hence, to their respective effectors) was also true for the *Pu* promoter, we carried out an equivalent experiment with the reporter strain *P. putida* SF05 (*P. putida* KT2442::mini-Tn*5* Sm *Pu-lacZ*), which contains a transcrip-

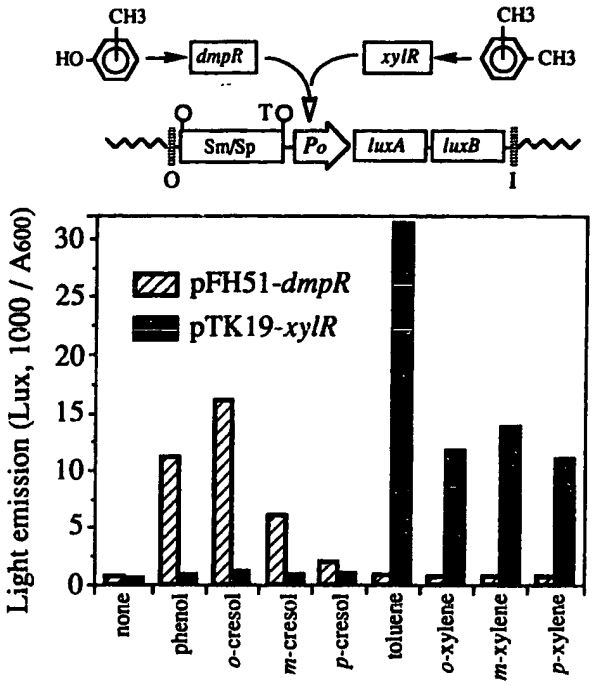

FIG. 2. Cross-activation of the *Po* promoter by XylR. The drawing at the top (not to scale) illustrates the structure of the hybrid mini-Tn*5* element containing the transcriptional fusion *Po-luxAB* inserted into the chromosome (zigzag lines) of *P. putida* 270 used in the experiment. The Sm^r/Sp^r selection marker of the hybrid transposon is indicated, as are the positions of the I and O ends of Tn*5*, the locations of transcriptional terminators (T, represented by circles on stalks) shielding the reporter system, and the directions of transcription of the *Po* promoter. For induction experiments, *P. putida* 270 cells harboring either pFH51 (*dmpR*$^+$) or pTK19 (*xylR*$^+$) were grown at 30°C in LB medium to an OD_{600} of 0.5 and exposed either to saturating vapors of toluene and xylenes (XylR effectors) or to 2.5 mM phenol and cresols (DmpR effectors). The cells were left for a further 2 h and then assayed for light emission. The results are the averages of triplicate determinations performed on each of two independent cultures. Note that the only activator present in the strain is from the plasmid.

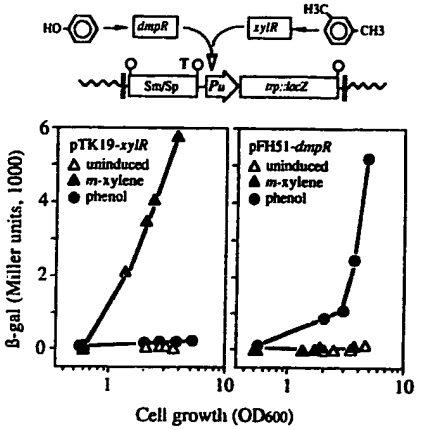

FIG. 3. Induction profile of the *Pu* promoter in response to DmpR- and XylR-specific effectors. The organization of the chromosomal insert containing the reporter *Pu-lacZ* transcriptional fusion of *P. putida* SF05 is indicated at the top. For the experiment shown, *P. putida* SF05 cells harboring either pFH51 (*dmpR*$^+$) or pTK19 (*xylR*$^+$) were grown to an OD_{600} of 0.5 and were subsequently exposed to either *m*-xylene vapor or 2.5 mM phenol as indicated. The course of β-Gal accumulation during subsequent growth was monitored for the next 16 h.

tional *Pu-lacZ* fusion in its chromosome. As shown in Fig. 3, *P. putida* SF05 (pFH51-*dmpR*$^+$) accumulated β-Gal in the presence of phenol but not when exposed to *m*-xylene, while *P. putida* SF05 (pTK19-*xylR*$^+$) was nonresponsive to phenol but, as expected, was strongly induced by its native effector, *m*-xylene. Figure 3 also shows that under the conditions used, full *Pu* induction was more dependent on growth phase when the promoter was regulated by DmpR than when it was activated by XylR.

The phenol-degrading pathway of *Pseudomonas* sp. strain CF600 is strongly induced by XylR in response to TOL effectors. To determine whether the cross-regulation of the *Po* promoter by XylR detected with the *luxAB* fusion would be significant under more physiological conditions, we directly examined the effect of XylR on the expression of the phenol-degrading pathway of *Pseudomonas* sp. strain CF600. For this, we monitored the *dmpB*-encoded C2,3O activity resulting from expression of the 8th gene of the *dmp* operon. The response of *Pseudomonas* sp. strain CF600 (with and without the pTK19 [*xylR*$^+$] plasmid) in the presence of different aromatic compounds was measured. The results, summarized in Fig. 4, show not only that XylR can substitute DmpR to make the phenol-degrading pathway responsive to toluene and *m*-xylene but also that under the conditions of the experiment, XylR can trigger expression of the products of the pathway to levels approximately fivefold higher than those elicited by the native DmpR regulator. Furthermore, *Pseudomonas* sp. strain CF600 harboring pTK19-*xylR*$^+$ was poorly induced by phenol, to about a third of the level of the same strain without the plasmid. This reduction is probably due to the nonproductive

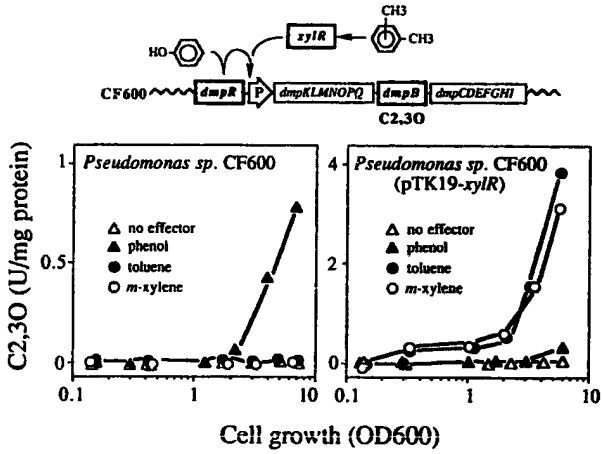

FIG. 4. Expression of C2,3O activity by *Pseudomonas* sp. strain CF600 with and without a plasmid expressing the *xylR* gene. The relevant elements involved in the expression of the *dmpB*-C2,3O gene from the *Po* promoter of the *dmp* operon are summarized at the top (not to scale). *Pseudomonas* sp. strain CF600 cells alone (left) or harboring pTK19 (*xylR*$^+$) (right) were grown to an OD_{600} of 0.1 and exposed to the aromatic effectors indicated in each case. The cells were then harvested at different time points and sonicated, and the resulting extracts were assayed for C2,3O activity as explained in Materials and Methods.

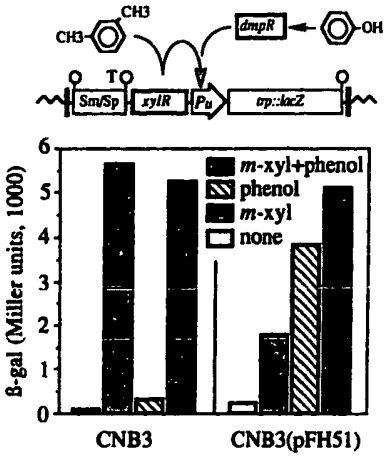

FIG. 5. Coinduction of *Pu* in the presence of XylR and DmpR effectors. The diagram at top represents the organization of the hybrid mini-Tn*5* transposon inserted into the chromosome of *P. putida* CNB3. *P. putida* CNB3 cells either alone (left) or containing pFH51 (*dmpR*$^+$) (right) were grown to an $OD_{(nm)}$ of 0.5 and induced with *m*-xylene, phenol, or both, as indicated. The bar diagram represents the accumulation of β-Gal after 16 h of exposure of the cultures to the different effectors.

occupation by XylR of coincident or nearby DmpR target sites within the *Po* promoter (see Discussion).

DmpR and XylR simultaneously activate the *Pu* promoter in response to phenol and *m*-xylene. Since an excess of *xylR* copy number with respect to that of *dmpR* repressed the induction of *Po* in response to phenol somewhat (see above), we asked whether coexpressed DmpR and XylR could influence *Pu* so that it could simultaneously respond to the presence of the effectors of both regulators. To examine this issue, we constructed the reporter strain *P. putida* CNB3 (*P. putida* KT2442::mini-Tn*5* XylR/*Pu-lacZ*) and introduced the pFH51 (*dmpR*$^+$) plasmid. The resulting strain was then subjected to the various coinduction assays with *m*-xylene and phenol as shown in Fig. 5. In this case, although the presence of the extra *dmpR* gene copies reduced the induction of *Pu* by *m*-xylene, the *P. putida* CNB3 (pFH51-*dmpR*$^+$) strain was similiariy responsive to both effectors either alone or in combination. Maximal expression levels were obtained only when the effectors of both regulators were present.

DmpR and XylR recognize the same DNA region in *Pu*. To determine whether the observed cross-regulation effects were due to binding of DmpR and XylR to the same cognate DNA sequences within the upstream activating regions of their respective promoters, or alternatively, whether they were due to an activation phenomenon independent of DNA binding (as has been observed with other regulators of the family [20]), we constructed isogenic *P. putida* KT2442 derivatives carrying *Pu-lacZ* fusions differing solely by the presence or absence of the upstream region known to be involved in XylR binding (1, 7, 21). Each of these strains was then transformed with either pTK19 (*xylR*$^+$) or pFH51 (*dmpR*$^+$) and subjected to induction assays with either phenol or *m*-xylene. As shown in Fig. 6, in the absence of the *Pu* region upstream from −106 with respect to the transcription initiation site, the *Pu* promoter became totally nonresponsive, irrespective of the presence of activators and their effectors. This result suggests that cross-activation requires actual binding of either DmpR or XylR to target sequences located in the same upstream region of *Pu*.

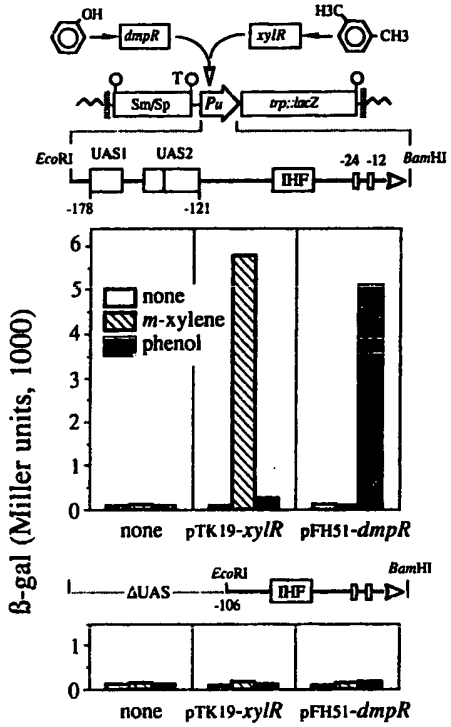

FIG. 6. Requirement of UAS for activation of *Pu* by XylR and DmpR. The relevant portions of the *Pu* promoter segments included in the two *lacZ* elements inserted in the chromosomes of isogenic strains *P. putida* SF05 (*Pu-lacZ*) and *P. putida* SF05Δ (*PuΔUAS-lacZ*) are shown above the appropriate bar diagram. Strains SF05 and SF05Δ alone or harboring either pTK19 (*xylR*$^+$) or pFH51 (*dmpR*$^+$) were induced overnight with *m*-xylene or phenol as indicated in each case. Under all conditions tested, the activity of the *PuΔUAS* promoter was negligible.

DISCUSSION

Both XylR and DmpR belong to the NtrC family of transcriptional activators (23, 30, 39) which characteristically exert their action in concert with RNA polymerase containing the alternative factor σ^{54} (σ^{54}-RNAP). These two proteins are virtually identical in size (63.7 and 63.3 kDa, respectively) and share a high degree of sequence identity throughout their entire lengths (>67%). Maximum similarity (>79%) is found within the central domain, which is thought to interact with the polymerase (30, 32). The amino-terminal A domains, which span the leading third of the proteins, have been shown by genetic means to directly interact with the aromatic effector (4, 41) and mediate inducer specificity. The A domains of DmpR and XylR share >64% similarity and can be swapped, thus completely shifting the effector specificity of the resulting chimeric activator (41). Despite the homology between the two regulators, the promoters they control transcribe different operons. The *Pu* promoter drives expression of a cluster of six cistrons involved in the bioconversion of toluene and *m*- and *p*-xylenes into benzoate and toluates, respectively (Fig. 1). The first functional gene of the *upper* TOL operon encodes benzaldehyde dehydrogenase (14). On the other hand, *Po* directs transcription of a large polycistronic operon of 15 genes in response to the presence of phenol or cresols (36, 42). The first genes of the operon encode the polypeptides of a multicomponent phenol hydroxylase (40). Therefore, the similarity of

the promoter regions does not extend into the first operonic genes.

Although both *Pu* and *Po* belong to the σ^{54}-dependent class of promoters, comparative analysis of the corresponding regions reveals that they are also considerably different. There is no significant nucleotide sequence similarity downstream of the binding sites for the σ^{54}-RNAP at positions −12 to −24. Furthermore, the *Pu* promoter has a functional integration host factor (IHF)-binding site between positions −52 and −79 which is absent (or placed elsewhere [Fig. 1]) in *Po*. However, a region of approximately 50 bp shows considerable sequence similarity between positions −124 and −175 of *Pu* and positions −123 and −174 of *Po* (Fig. 1C). These regions are also homologous to a segment (−136 to −187) upstream of *Ps*, the other XylR-dependent TOL promoter (11, 19) (Fig. 1A). The homologous region in *Pu*, *Po*, and *Ps* has been shown, in the case of *Pu*, to include the binding sites for XylR, as revealed by in vitro (7) and in vivo (1) footprinting.

The results shown in this paper demonstrate that XylR and DmpR recognize overlapping or nearby DNA sequences located within the same region of the *Pu* promoter and, most likely, within the *Po* promoter as well. Although we have not rigorously proven that the two activators recognize the same DNA sequence (see below), it is clear that the mutual interchange for the activation of each other's cognate promoter is not just a residual cross-talk but is a significant and highly specific cross-activation. Both regulators affect the promoters at comparable levels in vivo, and, in at least one case (Fig. 4), XylR stimulates the production of the catabolic enzymes for the degradation of phenol to much higher levels than those afforded by the native DmpR regulator of the system. However, as indicated by the results shown in Fig. 2 and 3, the operative binding of DmpR and XylR to both *Pu* and *Po* seems to be similar. In comparable situations, namely, those involving a chromosomal promoter-reporter fusion and the regulator cloned in an RSF1010 derivative, both *Pu* and *Po* respond to the two types of effectors (phenol and *m*-xylene) within the same range. An interesting observation resulting from the data shown in Fig. 4 and 5 is that when one of the two regulators is present at a gene copy number exceeding that of the other by about 20-fold (because of the plasmid vector used), the responses of both promoters to the low-abundance regulator are decreased. This may be due to the nonproductive occupation of some of the promoter-UAS sequences by the unactivated regulator which is in excess. This is consistent with our previous observation that XylR can bind *Pu* regardless of the presence of its effector (1, 7). Additional support for the idea of nonproductive occupancy of the promoters by unactivated regulators comes from the dual-effector induction experiment shown in Fig. 5. In this case, simultaneous exposure of coexpressed DmpR and XylR to effectors of both regulators resulted in the full activation of the *Pu-lacZ* reporter system. This result may simply reflect the net effect of *Pu* being occupied by a mixture of activated DmpR and XylR. Alternatively, there is the intriguing possibility that the activation is the consequence of formation of functional DmpR-XylR pairs.

A question raised by our results is the precise nature of the DNA sequence recognized by XylR and DmpR. The 20-amino-acid helix-turn-helix motifs located at the carboxyl ends of the two proteins and thought to constitute the actual DNA-binding domains (30) have 16 residues in common (39). XylR and DmpR may, therefore, recognize similar, even identical nucleotide sequences. DNase I footprints with XylR span a relatively long region of the *Pu* promoter (7) which includes an inverted repeat of the sequence 5′-TTGANC AAATC-3′. However, although XylR binds with a defined pattern of interactions throughout the −121-to−178 region of *Pu*, the actual sequence recognized by XylR ultimately remains undefined. Shorter motifs appear repeatedly throughout the 52-bp segment of maximum nucleotide sequence similarity in *Pu*, *Po*, and *Ps* (Fig. 1C), such as 5′-TTGNNCAA-3′, 5′-TTGAT-3′, or 5′-CAAATC-3′. Interestingly, regardless of the sequence used as a reference for comparisons, *Po* systematically resembles *Pu* and *Ps* to a greater extent than *Pu* and *Ps* resemble each other. These results support the idea that the regulators of the NtrC family (such as DmpR and XylR) have the potential to coevolve along with their target sequences as regulatory cassettes which can eventually be recruited by different operons. Perhaps the basic unit includes the activator gene constitutively and divergently expressed from the σ^{54}-dependent promoter that it regulates, as is the case with *Po* and *Ps* (Fig. 1). Other gene clusters could then come under the same regulatory control by simple acquisition of a properly positioned UAS.

DNA and amino acid sequence comparisons among the members of the NtrC family of proteins (30) have revealed that their functional domains are arranged in modules, the most invariant of which is the central portion of the proteins thought to interact directly with the σ^{54}-RNAP. However, phylogenetic relationships among the leading amino-terminal domains (involved in signal reception) and the carboxyl-terminal domains (DNA binding) indicate that the functionally distinct domains have evolved quite independently of each other and of the central portions of the proteins. For instance, the C-terminal domain of NtrC is related to the factor for inversion stimulation (FIS protein) of *E. coli* (32). Unlike NtrC, however, the sequences of the carboxyl-terminal domains of XylR of *P. putida* and AlgB of *Pseudomonas aeruginosa* are much more similar than are their central domains, thus suggesting that the DNA-binding domains of these proteins (but not the other portions of the polypeptide) have the same and perhaps more recent origin within the *Pseudomonas* genus (30). These observations imply that both coding and regulatory sequences must be shuffled among distant locations of the genetic complement during their evolutionary divergence (9, 13, 16, 35, 38, 43).

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REFERENCES

1. **Abril, M. A., M. Buck, and J. L. Ramos.** 1991. Activation of the *Pseudomonas* TOL plasmid *upper* pathway operon: identification of binding sites for the positive regulator XylR and for integration host factor protein. J. Biol. Chem. **266**:5832–5838.
2. **Abril, M. A., C. Michan, K. N. Timmis, and J. L. Ramos.** 1989. Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. J. Bacteriol. **171**:6782–6790.
3. **Assinder, S. J., and P. A. Williams.** 1990. The TOL plasmids: determinants of the catabolism of toluene and xylenes. Adv. Microb. Physiol. **31**:1–69.
4. **Delgado, A., and J. L. Ramos.** 1994. Genetic evidence for activation of the positive transcriptional regulator XylR, a member of

the NtrC family of regulators, by effector binding. J. Biol. Chem. 269:8059–8062.
5. **de Lorenzo, V., S. Fernández, M. Herrero, U. Jacubzik, and K. N. Timmis.** 1993. Engineering alkyl- and haloaromatic-responsive gene expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*. Gene 130:41–46.
6. **de Lorenzo, V., M. Herrero, U. Jacubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. 172:6568–6572.
7. **de Lorenzo, V., M. Herrero, M. Metzke, and K. N. Timmis.** 1991. An upstream XylR- and IHF-induced nucleoprotein complex regulates the σ^{54}-dependent *Pu* promoter of TOL plasmid. EMBO J. 10:1159–1167.
8. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. Methods Enzymol. 235:386–405.
9. **Deretic, V., W. M. Konyecsni, C. D. Mohr, D. W. Martin, and N. S. Hibler.** 1989. Common denominators of promoter control in *Pseudomonas* and other bacteria. Bio/Technology 7:1249–1254.
10. **Franklin, F. C. H.** 1985. Broad host range cloning vectors for gram-negative bacteria, p. 165–184. *In* D. M. Glover (ed.), DNA cloning—a practical approach, vol. 1. IRL Press, Oxford.
11. **Gomada, M., S. Inouye, H. Imaishi, A. Nakazawa, and T. Nakazawa.** 1992. Analysis of an upstream regulatory sequence required for activation of the regulatory gene *xylS* in xylene metabolism directed by the TOL plasmid of *Pseudomonas putida*. Mol. Gen. Genet. 233:419–426.
12. **Harayama, S., M. Kok, and E. L. Neidle.** 1992. Functional and evolutionary relationships between diverse oxygenases. Annu. Rev. Microbiol. 46:565–601.
13. **Harayama, S., M. Rekik, A. Bairoch, E. L. Neidle, and L. N. Ornston.** 1991. Potential DNA slippage structures acquired during evolutionary divergence of *Acinetobacter calcoaceticus* chromosomal *benABC* and *Pseudomonas putida* TOL pWW0 plasmid *xylXYZ*, genes encoding benzoate dioxygenases. J. Bacteriol. 173: 7540–7548.
14. **Harayama, S., M. Rekik, M. Wubbolts, M. Rose, R. A. Leppik, and K. N. Timmis.** 1989. Characterization of five genes in the *upper* pathway operon of TOL plasmid pWW0 from *Pseudomonas putida* and identification of the gene products. J. Bacteriol. 171:5048–5055.
15. **Harayama, S., and K. N. Timmis.** 1992. Aerobic biodegradation of aromatic hydrocarbons by bacteria, p. 99–156. *In* H. Sigel and A. Sigel (ed.), Metal ions in biological systems, vol. 28. Marcel Dekker Inc., New York.
16. **Hartnett, C. S., E. L. Neidle, K. L. Ngai, and L. N. Ornston.** 1990. DNA sequences of genes encoding *Acinetobacter calcoaceticus* protocatechuate 3,4-dioxygenase: evidence indicating shuffling of genes and of DNA sequences within genes during their evolutionary divergence. J. Bacteriol. 172:956–966.
17. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. 172:6557–6567.
18. **Hofer, B., L. D. Eltis, D. N. Dowling, and K. N. Timmis.** 1993. Genetic analysis of a *Pseudomonas* locus encoding a pathway for biphenyl/polychlorinated biphenyl degradation. Gene 130:47–55.
19. **Holtel, A., K. N. Timmis, and J. L. Ramos.** 1992. Upstream binding sequences of the XylR activator protein and integration host factor in the *xylS* gene promoter region of *Pseudomonas* TOL plasmid. Nucleic Acids Res. 20:1755–1762.
20. **Huala, E., and F. M. Ausubel.** 1989. The central domain of *Rhizobium meliloti* NifA is sufficient to activate transcription from the *R. meliloti nifH* promoter. J. Bacteriol. 171:3354–3365.
21. **Inouye, S., M. Gomada, U. M. X. Sangodkar, A. Nakazawa, and T. Nakazawa.** 1990. Upstream regulatory sequences for transcriptional activator XylR in the first operon of xylene metabolism of the TOL plasmid. J. Mol. Biol. 216:251–260.
22. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1987. Expression of the regulatory gene *xylS* on the TOL plasmid is positively controlled by the *xylR* gene product. Proc. Natl. Acad. Sci. USA 84:5182–5186.
23. **Inouye, S., A. Nakazawa, and T. Nakazawa.** 1988. Nucleotide sequence of the regulatory gene *xylR* of the TOL plasmid from *Pseudomonas putida*. Gene 66:301–306.
24. **Kessler, B., V. de Lorenzo, and K. N. Timmis.** 1992. A general system to integrate *lacZ* fusions into the chromosomes of gram-negative eubacteria: regulation of the *Pm* promoter of the TOL plasmid studied with all controlling elements in monocopy. Mol. Gen. Genet. 233:293–301.
25. **Köhler, T., S. Harayama, J. L. Ramos, and K. N. Timmis.** 1989. Involvement of *Pseudomonas putida* RpoN σ factor in regulation of various metabolic functions. J. Bacteriol. 171:4326–4333.
26. **Kunkel, T., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-directed mutagenesis without phenotypic selection. Methods Enzymol. 154:367–382.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Marques, S., and J. L. Ramos.** 1993. Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. Mol. Microbiol. 9:923–929.
29. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Morett, E., and L. Segovia.** 1993. The σ^{54} bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. J. Bacteriol. 175:6067–6074.
31. **Myers, R. M., L. S. Lerman, and T. Maniatis.** 1985. A general procedure for saturation mutagenesis of cloned DNA fragments. Science 229:242–247.
32. **North, A., K. E. Klose, K. M. Stedman, and S. Kustu.** 1993. Prokaryotic enhancer-binding proteins reflect eukaryote-like modularity: the puzzle of nitrogen regulatory protein C. J. Bacteriol. 175:4267–4273.
33. **Nozaki, M.** 1970. Metapyrocatechase (*Pseudomonas*). Methods Enzymol. 17:522–525.
34. **Olsson, O., C. Konez, and A. A. Szalay.** 1988. The use of the *luxA* gene of the bacterial luciferase operon as a reporter gene. Mol. Gen. Genet. 215:1–9.
35. **Ornston, L. N., and W. K. Yeh.** 1979. Origins of metabolic diversity: evolutionary divergence by sequence repetition. Proc. Natl. Acad. Sci. USA 76:3996–4000.
36. **Powlowski, J., and V. Shingler.** Genetics and biochemistry of phenol degradation by *Pseudomonas*. Biodegradation, in press.
37. **Ramos, J. L., A. Stolz, W. Reineke, and K. N. Timmis.** 1986. Altered effector specificities in regulators of gene expression: TOL plasmid *xylS* mutants and their use to engineer expansion of the range of aromatic degraded by bacteria. Proc. Natl. Acad. Sci. USA 83:8467–8471.
38. **Shapiro, J. A.** 1993. Natural genetic engineering of the bacterial genome. Curr. Opin. Genet. Dev. 3:845–848.
39. **Shingler, V., M. Bartilson, and T. Moore.** 1993. Cloning and nucleotide sequence of the gene encoding the positive regulator (DmpR) of the phenol catabolic pathway encoded by pVI150 and identification of DmpR as a member of the NtrC family of transcriptional activators. J. Bacteriol. 175:1596–1604.
40. **Shingler, V., F. C. H. Franklin, M. Tsuda, D. Holroyd, and M. Bagdasarian.** 1989. Molecular analysis of a plasmid encoded phenol hydroxylase of *Pseudomonas* CF600. J. Gen. Microbiol. 135:1083–1092.
41. **Shingler, V., and T. Moore.** 1994. Sensing aromatic compounds by the DmpR transcriptional activator of phenol-catabolizing *Pseudomonas* sp. strain CF600. J. Bacteriol. 176:1555–1560.
42. **Shingler, V., J. Powlowski, and U. Marklund.** 1992. Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. J. Bacteriol. 174:711–724.
43. **Yeh, W. K., and L. N. Ornston.** 1980. Origins of metabolic diversity: substitutions of homologous sequences into genes for enzymes with different catalytic activities. Proc. Natl. Acad. Sci. USA 143:5365–5369.